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*J Clin Invest.* 1988;82(1):168-176. <https://doi.org/10.1172/JCI113566>.

### Research Article

Calcium has been implicated as an important factor in prostaglandin production. Phospholipase A2, the enzyme believed to be rate limiting for prostaglandin synthesis, is stimulated by Ca<sup>2+</sup>; however, the levels of Ca<sup>2+</sup> necessary to stimulate phospholipase A2 in cell-free systems are higher than levels achieved in intact cells in response to agonists that stimulate prostaglandin synthesis. We examined the calcium dependency of prostaglandin E2 (PGE2) synthesis in the glomerular mesangial cell. Vasopressin enhanced PGE2 synthesis by mechanisms independent of extracellular Ca<sup>2+</sup> concentration. The Ca<sup>2+</sup> concentration dependency of PGE2 production was established by rendering cells permeable with digitonin and clamping Ca<sup>2+</sup> concentration at various levels. When cytosolic free Ca<sup>2+</sup> concentration ([Ca<sup>2+</sup>]<sub>f</sub>) was set at levels equal to those measured after stimulation with vasopressin in the intact cell, the PGE2 production by the Ca<sup>2+</sup>-clamped permeabilized cells was approximately one-half of that obtained in nonpermeabilized cells stimulated with vasopressin. Since stimulation of mesangial cells with vasopressin increases protein kinase C activation as well as [Ca<sup>2+</sup>]<sub>f</sub> the effects on PGE2 production of protein kinase C activation with phorbol myristate acetate (PMA) were examined. When permeabilized cells were exposed to Ca<sup>2+</sup> concentrations in the range of [Ca<sup>2+</sup>]<sub>f</sub> measured in cells treated with vasopressin the addition of PMA approximately doubled PGE2 production. No increase in PGE2 production was observed with PMA when Ca<sup>2+</sup> concentration was fixed [...]

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# Calcium Dependency of Prostaglandin E<sub>2</sub> Production in Rat Glomerular Mesangial Cells

## Evidence That Protein Kinase C Modulates the Ca<sup>2+</sup>-dependent Activation of Phospholipase A<sub>2</sub>

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### Abstract

Calcium has been implicated as an important factor in prostaglandin production. Phospholipase A<sub>2</sub>, the enzyme believed to be rate limiting for prostaglandin synthesis, is stimulated by Ca<sup>2+</sup>; however, the levels of Ca<sup>2+</sup> necessary to stimulate phospholipase A<sub>2</sub> in cell-free systems are higher than levels achieved in intact cells in response to agonists that stimulate prostaglandin synthesis. We examined the calcium dependency of prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) synthesis in the glomerular mesangial cell. Vasopressin enhanced PGE<sub>2</sub> synthesis by mechanisms independent of extracellular Ca<sup>2+</sup> concentration. The Ca<sup>2+</sup> concentration dependency of PGE<sub>2</sub> production was established by rendering cells permeable with digitonin and clamping Ca<sup>2+</sup> concentration at various levels. When cytosolic free Ca<sup>2+</sup> concentration ([Ca<sup>2+</sup>]<sub>i</sub>) was set at levels equal to those measured after stimulation with vasopressin in the intact cell, the PGE<sub>2</sub> production by the Ca<sup>2+</sup>-clamped permeabilized cells was approximately one-half of that obtained in nonpermeabilized cells stimulated with vasopressin. Since stimulation of mesangial cells with vasopressin increases protein kinase C activation as well as [Ca<sup>2+</sup>]<sub>i</sub>, the effects on PGE<sub>2</sub> production of protein kinase C activation with phorbol myristate acetate (PMA) were examined. When permeabilized cells were exposed to Ca<sup>2+</sup> concentrations in the range of [Ca<sup>2+</sup>]<sub>i</sub> measured in cells treated with vasopressin the addition of PMA approximately doubled PGE<sub>2</sub> production. No increase in PGE<sub>2</sub> production was observed with PMA when Ca<sup>2+</sup> concentration was fixed at basal levels of < 100 nM. Ca<sup>2+</sup>-dependent acylhydrolase activity and PGE<sub>2</sub> production were inhibited by calmodulin inhibitors, W-7 and compound 48/80. Thus, vasopressin-induced PGE<sub>2</sub> production could be explained by a synergistic effect of protein kinase C activation together with an increase in [Ca<sup>2+</sup>]<sub>i</sub>. A synergistic action of Ca<sup>2+</sup> and PMA on acylhydrolase activity could also be observed in nonpermeabilized cells where A23187 was used to increase [Ca<sup>2+</sup>]<sub>i</sub>. The effect of PMA was mimicked by another stimulant of protein kinase C, 1-oleoyl 2-acetylgllycerol, albeit with lower potency. Neither PMA nor 1-oleoyl 2-acetylgllycerol alone had any effect on

acylhydrolase activity. Vasopressin, in the presence of GTPγS, stimulated phospholipase C in permeabilized cells when [Ca<sup>2+</sup>]<sub>i</sub> was fixed at < 100 nM, without an associated increase in acylhydrolase activity. This evidence, together with inhibition of acylhydrolase activity with phospholipase A<sub>2</sub> inhibitors, dibucaine and mepacrine, indicates that the primary acylhydrolase activity was due to phospholipase A<sub>2</sub>. The enhanced phospholipase A<sub>2</sub> activity observed with protein kinase C activation when [Ca<sup>2+</sup>]<sub>i</sub> is increased may be related to phosphorylation of phospholipase A<sub>2</sub> itself or phospholipase A<sub>2</sub> modulatory proteins. These experiments demonstrate that both Ca<sup>2+</sup> and protein kinase C play important roles in the regulation of phospholipase A<sub>2</sub> and PGE<sub>2</sub> synthesis.

### Introduction

The mechanisms of regulation of prostaglandin production are incompletely understood. Calcium has been implicated as a primary factor in the regulation of prostaglandin synthesis since it stimulates many purified acylhydrolases including phospholipase A<sub>2</sub>, the enzyme generally believed to be rate limiting for prostaglandin production. The Ca<sup>2+</sup> sensitivity of phospholipase A<sub>2</sub> activity, however, may not be sufficient to explain this enzyme's activation in the intact cell exposed to stimuli such as vasopressin. The level of Ca<sup>2+</sup> concentration necessary to stimulate phospholipase activity in various assay systems is higher than the levels of cytosolic free Ca<sup>2+</sup> concentration ([Ca<sup>2+</sup>]<sub>i</sub>)<sup>1</sup> achieved even in stimulated cells (1-3). It is possible that the in vitro assay systems for enzymatic activity may result in incomplete expression of phospholipase A<sub>2</sub> activity because of the absence of important cofactors or the enhanced expression of endogenous inhibitor activity (3). Alternatively, other modulatory processes that enhance Ca<sup>2+</sup>-dependent phospholipase A<sub>2</sub> activity in the cell may be absent in phospholipase A<sub>2</sub> assay systems involving cell-free extracts. We designed experiments to define the Ca<sup>2+</sup> dependence of phospholipase A<sub>2</sub> activity under conditions devised to maintain the enzyme in its natural environment (i.e., the cell). This characterization then allowed us to evaluate the importance of changes in [Ca<sup>2+</sup>]<sub>i</sub> for phospholipase A<sub>2</sub> activation and prostaglandin synthesis observed with hormonal stimulation.

Many agonists that increase prostaglandin synthesis in the mesangial cell also activate phospholipase C (4). This results in an increase in both inositol trisphosphate and diacylglycerol levels. The former releases Ca<sup>2+</sup> from intracellular storage sites increasing [Ca<sup>2+</sup>]<sub>i</sub> (4, 5) and the latter stimulates protein kinase C. Since an increase in [Ca<sup>2+</sup>]<sub>i</sub> alone was not sufficient to

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The data were presented in part at the meeting of the American Society for Chemical Research and published in part in abstract form in *Clin. Res.* 34: 611, 1986.

Received for publication 13 August 1987 and in revised form 8 January 1988.

J. Clin. Invest.

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0021-9738/88/07/0168/09 \$2.00

Volume 82, July 1988, 168-176

1. Abbreviations used in this paper: AVP, vasopressin [Ca<sup>2+</sup>]<sub>i</sub>, cytosolic free Ca<sup>2+</sup> concentration; GTPγS, guanosine 5'-O-(3-thiotriphosphate); PMA, phorbol-12-myristate 13-acetate; TMB-8, 8-(N,N-diethylamino)-octyl-3,4,5-trimethoxybenzoate hydrochloride; W-7, N-(6-aminoethyl)-L-naphthelenesulfonamide.

explain prostaglandin production in our experiments, we examined whether protein kinase C played an important role in this process.

To understand the role of  $\text{Ca}^{2+}$  and protein kinase C in the mediation of prostaglandin synthesis in the glomerular mesangial cell we asked the following questions: (a) Does  $\text{PGE}_2$  synthesis in the intact mesangial cell depend upon  $\text{Ca}^{2+}$  entry from the external milieu or release of  $\text{Ca}^{2+}$  from intracellular storage sites? (b) Is  $\text{PGE}_2$  production a direct function of changes in cytosolic free  $\text{Ca}^{2+}$  and does phospholipase  $\text{A}_2$  activity and  $\text{PGE}_2$  synthesis depend upon calmodulin? (c) Does protein kinase C modulate the  $\text{Ca}^{2+}$ -dependent activation of phospholipase  $\text{A}_2$  and  $\text{PGE}_2$  production?

## Methods

**Cultured mesangial cells.** Glomeruli were isolated from Sprague-Dawley rats using a graded-sieve technique and mesangial cells grown as previously described (5, 6). Mesangial cells were carried in 100 mm plastic dishes (Falcon Labware, Oxnard, CA) in RPMI 1640 tissue culture medium (Gibco, Grand Island, NY) with 20% FCS without antibiotics. The medium was maintained at pH 7.2–7.4. Cells were used in passages 30–50. Cells were passaged using split ratios of 1:5 at 7–10-d intervals, maintained in an incubator at 37°C, and aerated with 95% air, 5%  $\text{CO}_2$ . Media was changed 24 h before the experiments.

**Stimulation of prostaglandin  $E_2$  synthesis.** Media was removed from cells by washing twice and cells were then incubated with a buffer containing NaCl, 145 mM; KCl, 5 mM;  $\text{MgSO}_4$ , 0.5 mM;  $\text{NaHPO}_4$ , 1 mM; glucose, 5 mM; Hepes, 20 mM; with various concentrations of  $\text{CaCl}_2$  with or without EGTA, pH 7.4. Agonist (vasopressin, A23187, TMB-8, phorbol myristate acetate, PMA) or digitonin was then added to cells for 10 min. In some experiments the extracellular  $[\text{Ca}^{2+}]$  was reduced to 1–10  $\mu\text{M}$ , by not adding any exogenous  $\text{Ca}^{2+}$  to the buffer, or < 1.0  $\mu\text{M}$  by adding varying amounts of EGTA to nominally  $\text{Ca}^{2+}$ -free buffers. Samples of incubation buffer were taken prior to and 10 min subsequent to the addition of agonist.  $\text{PGE}_2$  was determined by RIA.

**Cytosolic free calcium concentration.** Cells were loaded with fura-2 using techniques previously described (5). Cells were lifted off the plastic support by 5–7 min exposure to  $\text{Ca}^{2+}$ ,  $\text{Mg}^{2+}$ -free HBSS containing 0.5 g trypsin (1:250) and 0.2 g EDTA/liter. Cells were resuspended in a modified Krebs-Henseleit bicarbonate buffer containing: NaCl, 120 mM; KCl, 2.7 mM;  $\text{MgSO}_4$ , 1.4 mM;  $\text{KH}_2\text{PO}_4$ , 1.4 mM;  $\text{NaHCO}_3$ , 25 mM (pH 7.4) containing 0.5 mM  $\text{CaCl}_2$ , 10 mM glucose, 20 mM Hepes and 1.5% gelatin for 30 min. They were loaded with fura-2 by incubating in identical buffer with the acetylmethoxy ester of fura-2 (2.5  $\mu\text{M}$ ) for 20–60 min. At the end of the loading period > 99% of cells excluded trypan blue. Prior to fluorescence measurement, cells were washed by centrifugation twice and resuspended at the concentration of  $3\text{--}6 \times 10^6$  cells/ml in 2 ml of buffer containing: NaCl, 145 mM; KCl, 5 mM;  $\text{MgSO}_4$ , 0.5 mM;  $\text{Na}_2\text{HPO}_4$ , 1 mM; glucose, 5 mM; Hepes, 20 mM; and  $\text{CaCl}_2$ , 0.5 mM. Fluorescence was measured at 37°C using an Aminco-Bowman spectrofluorimeter with a temperature-controlled cuvette holder employing techniques previously described (5, 7). Calibration of the fluorescence signal was performed using the previously described methods (5).

**Extracellular  $[\text{Ca}^{2+}]$  measurement.** The free extracellular  $[\text{Ca}^{2+}]$  concentration was measured with a  $\text{Ca}^{2+}$  selective electrode. Electrodes were constructed according to the methods of Affolter and Sigel (8) as modified by Prentki et al. (9). Calibration of the electrode was carried out by the method described by Bers (10). Free  $[\text{Ca}^{2+}]$  below  $10^{-5}$  M in the calibration solution was set by a  $\text{Ca}^{2+}$ -EGTA buffer system. The apparent association constant of EGTA ( $K_a$ ) for  $\text{Ca}^{2+}$  and the total amount of EGTA were determined in the actual calibration solutions used.  $K_a$  was determined to be  $4.2 \pm 0.5 \times 10^6 \text{ M}^{-1}$  and actual EGTA concentration in calibration solutions,  $966 \pm 17 \mu\text{M}$  ( $n = 8$ ). Calibration

solutions were at the same pH, temperature, and ionic strength as the cell incubation solutions.

**Free arachidonic acid and diglyceride determinations.** Mesangial cells were incubated with [ $^3\text{H}$ ]arachidonic acid (0.5  $\mu\text{Ci}$  in 10 ml of RPMI medium with 20% fetal calf serum) for 72 h before the experiment. Just prior to initiation of the experimental protocol the medium was removed and cells washed with buffer containing NaCl, 145 mM; KCl, 5 mM;  $\text{MgSO}_4$ , 0.5 mM;  $\text{Na}_2\text{HPO}_4$ , 1 mM;  $\text{CaCl}_2$ , 0.5 mM; glucose, 5 mM; fatty acid free bovine serum albumin, 200 mg/100 ml; buffered with 20 mM of Hepes to pH 7.4. Cells were then incubated in this buffer with 100 mg/100 ml albumin at 37°C. Digitonin and/or agonist were then added. After 10 min the buffer was rapidly transferred and the cells quenched in 2 ml of cold (4°C) methanol. The cells were then scraped and transferred to the collection tube containing the cell supernatant and formic acid (final concentration 0.2%). Plates were again scraped and solution transferred to the collection tube. By combining the cell supernatant with lysed cell fraction the [ $^3\text{H}$ ]arachidonate released into the media was combined with that present within the cells. 4 ml of chloroform/methanol (1:1.2) was added to the collection tube followed by 2 ml of chloroform. After vortexing and centrifuging samples to separate the phases the organic phase was dried under  $\text{N}_2$ . The dried samples were then dissolved in chloroform and spotted onto a heat-activated LK 5 DF silica gel plate (Whatman Ltd., Clifton, NJ) and developed with the organic phase of a mixture of ethylacetate/isooctane/glacial acetic acid/ $\text{H}_2\text{O}$  (55:75:8:100). Standards were cochromatographed, zones identified by staining with iodine vapor, and scraped into scintillation vials. After the addition of Dimilume Scintillation fluid the samples were counted in a scintillation counter (Hewlett-Packard, Palo Alto, CA). Total radioactivity introduced onto the TLC plate ranged from 20,000 to 40,000 cpm representing approximately one-third of the total organic phase radioactivity. Arachidonic acid and diglyceride in nonstimulated states each generally represented 1–3% of total TLC plate radioactivity.

**Materials.** Arginine vasopressin, phorbol 12-myristate 13-acetate (PMA), calmodulin, *N*-(6-aminohexyl)-1-naphthalenesulfonamide (W-7), compound 48/80, dibucaine, mepacrine, and guanosine 5'-O-(3-thiotriphosphate), GTP $\gamma$ S, were obtained from Sigma Chemical Co., St. Louis, MO. 8-(*N,N*-diethylamino)-octyl-3,4,5-trimethoxybenzoate hydrochloride (TMB-8), 4-Br A23187 and A23187 were purchased from Calbiochem-Behring Corp., La Jolla, CA.  $\text{PGE}_2$  antibody and [ $^3\text{H}$ ]arachidonate (sp act 80 Ci/mmol) was obtained from New England Nuclear Research Products, Boston, MA. 1-Oleoyl 2-acetylgllycerol, fura-2 AM and fura-2 free acid were purchased from Molecular Probes, Junction City, OR.

**Statistics.** Values are expressed as mean  $\pm$  1 SE. Groups were compared by paired or unpaired Student's *t* test or analysis of variance as appropriate.

## Results

**Effect of extracellular  $[\text{Ca}^{2+}]$  on  $\text{PGE}_2$  synthesis.**  $\text{PGE}_2$  production in response to vasopressin (100 nM) was examined in cells incubated with three different ranges of extracellular  $\text{Ca}^{2+}$  concentration in order to examine the effect of extracellular  $\text{Ca}^{2+}$  on this response. As indicated in Fig. 1 the increase in  $\text{PGE}_2$  observed with vasopressin was unaltered by reducing extracellular  $[\text{Ca}^{2+}]$  from 1.5 mM to 1–10  $\mu\text{M}$  or < 0.6  $\mu\text{M}$ . These data indicate that vasopressin-induced  $\text{PGE}_2$  synthesis is independent of the  $\text{Ca}^{2+}$  gradient across the cell plasma membrane and suggests that, if  $\text{Ca}^{2+}$  is involved in the response, it is  $\text{Ca}^{2+}$  that is released from intracellular storage sites rather than  $\text{Ca}^{2+}$ , which enters from the external milieu.

**Effect of TMB-8 on vasopressin-stimulated increase in  $[\text{Ca}^{2+}]_i$  and  $\text{PGE}_2$  synthesis.** To further examine whether release of  $\text{Ca}^{2+}$  from intracellular stores was responsible for  $\text{PGE}_2$  production, the effects of TMB-8 (30  $\mu\text{M}$ ), a putative

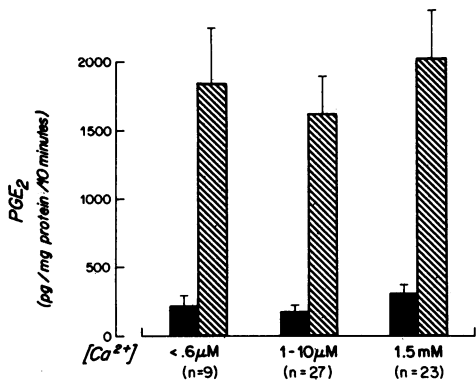


Figure 1. Prostaglandin  $E_2$  production in intact mesangial cells in response to 100 nM vasopressin (AVP) at various levels of extracellular  $[Ca^{2+}]$ . Reducing extracellular  $[Ca^{2+}]$  from 1.5 mM to 1–10  $\mu\text{M}$  or to levels below 0.6  $\mu\text{M}$  had no effect on the production of  $PGE_2$  induced with AVP. ■, basal; ▨, AVP stimulated.

inhibitor of  $Ca^{2+}$  release from intracellular stores (11), on vasopressin-induced  $PGE_2$  production were determined. The effect of TMB-8 on the vasopressin-induced increase in cytosolic free  $[Ca^{2+}]_i$  ( $[Ca^{2+}]_i$ ) is presented in Fig. 2. TMB-8 decreased the vasopressin-induced calcium response when compared to vasopressin treatment alone. TMB-8 also reduced vasopressin-stimulated  $PGE_2$  production. Mesangial cells were preincubated for 10 min with or without TMB-8 (30  $\mu\text{M}$ , vehicle  $H_2O$ ) before administration of vasopressin at extracellular calcium concentration of 1–10  $\mu\text{M}$  or 1.5 mM (Fig. 3). The relative decrease in  $PGE_2$  production was equivalent at both extracellular  $Ca^{2+}$  concentrations tested. While the effect of TMB-8 to decrease  $PGE_2$  production may be related to its effect on the vasopressin-induced  $Ca^{2+}$  increase the data to this point do not establish causality between these two effects of TMB-8. To examine whether TMB-8 altered  $PGE_2$  production by mechanisms independent of its effect on  $[Ca^{2+}]_i$  cells were permeabilized in the presence of TMB-8 and  $PGE_2$  production measured with  $[Ca^{2+}]$  fixed at two different levels (Fig. 4). TMB-8 reduced  $PGE_2$  production when  $[Ca^{2+}]$  was fixed within the ranges 0.1–5  $\mu\text{M}$  or 40–100  $\mu\text{M}$ . For each individ-

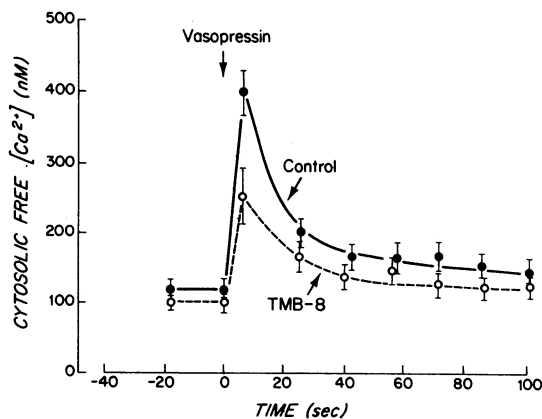


Figure 2. Effect of TMB-8 on vasopressin-induced increase in cytosolic free calcium concentration. TMB-8 (30  $\mu\text{M}$ ) significantly reduced the peak level of rise in cytosolic  $[Ca^{2+}]_i$  after vasopressin. Each tracing represents a composite of 13 experiments.

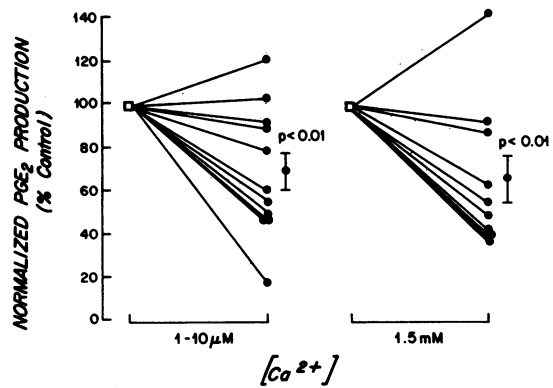


Figure 3. Effect of TMB-8 (30  $\mu\text{M}$ , ●) on  $PGE_2$  production in intact cells stimulated with vasopressin (100 nM) at extracellular  $[Ca^{2+}]$  of 1–10  $\mu\text{M}$  or 1.5 mM. In either case TMB-8 inhibited vasopressin-induced  $PGE_2$  production when compared with matched control cells stimulated with vasopressin in the absence of TMB-8 (□).

ual experiment the  $Ca^{2+}$  concentration of the control cells was equal to that of the TMB-8-treated cells. Therefore, the reduced  $PGE_2$  production measured in the presence of TMB-8 in intact cells was likely related to both a reduction in release of  $Ca^{2+}$  from intracellular stores as well as an effect independent of  $Ca^{2+}$  concentration. TMB-8 was thus not specific enough in its action to allow us to establish the  $Ca^{2+}$  dependency of  $PGE_2$  production.

*Ca<sup>2+</sup> and calmodulin dependency of PGE<sub>2</sub> synthesis and acylhydrolase activity.* If the increase in  $[Ca^{2+}]_i$  observed with vasopressin was responsible for the  $PGE_2$  production, it should be possible to demonstrate that the levels of  $[Ca^{2+}]_i$  achieved with vasopressin are sufficient to activate the cellular processes responsible for  $PGE_2$  production. To determine whether  $PGE_2$  production was a direct function of changes in  $[Ca^{2+}]_i$ ,  $PGE_2$  synthesis was determined in cells rendered permeable with digitonin (75  $\mu\text{g}/\text{ml}$ ) with  $Ca^{2+}$  concentration fixed at a given level ranging from less than 100 nM to 100  $\mu\text{M}$ . The  $Ca^{2+}$  concentrations were measured directly with a  $Ca^{2+}$  electrode.

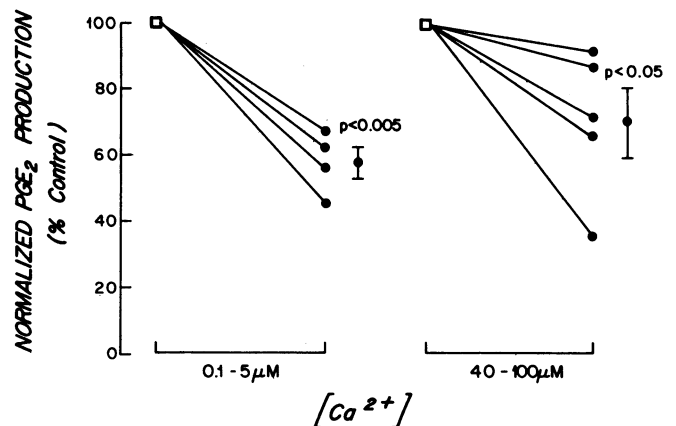


Figure 4. Effect of TMB-8 (●) on  $PGE_2$  production by cells made permeable with digitonin in the presence of  $Ca^{2+}$  concentrations over two ranges. In all cases the control cells (□) were exposed to the same  $Ca^{2+}$  concentration as the TMB-8-treated cells. TMB-8 (30  $\mu\text{M}$ ) decreased  $PGE_2$  production by a  $Ca^{2+}$ -independent mechanism.

There is a progressive increase in PGE<sub>2</sub> synthesis as the cytosolic [Ca<sup>2+</sup>]<sub>i</sub> is increased over this range (Fig. 5). To determine the calmodulin dependency of this process, the effect of a calmodulin inhibitor, compound 48/80 (100 μg/ml) (12), on PGE<sub>2</sub> production was examined. This agent significantly reduced PGE<sub>2</sub> synthesis at all levels of [Ca<sup>2+</sup>]<sub>i</sub> tested. Adding calmodulin (5 μg/ml) had no effect, indicating that calmodulin is not depleted by digitonin treatment.

To confirm that the Ca<sup>2+</sup> dependency of PGE<sub>2</sub> production was associated with a corresponding increase in acylhydrolase activity the release of arachidonic acid was examined in cells prelabeled with [<sup>3</sup>H]arachidonic acid and exposed to digitonin in the presence of [Ca<sup>2+</sup>]<sub>i</sub> of < 100 nM, 1–10 μM, or 0.5 mM. Free [<sup>3</sup>H]arachidonate represented 1.7±0.3, 5.4±1.9 and 14.9±7.0 (*n* = 4) percent, respectively, of total radioactivity in the lipid fraction as indicated in Table I. There was also an increase in diglyceride levels with increasing [Ca<sup>2+</sup>]<sub>i</sub>, as presented in Table I. The increases in [<sup>3</sup>H]arachidonate or [<sup>3</sup>H]diglyceride observed with increasing [Ca<sup>2+</sup>]<sub>i</sub> were not prevented by coincubation with indomethacin 1 μg/ml (data not shown). To further demonstrate the calmodulin dependency of the acylhydrolase activation we performed similar studies examining free [<sup>3</sup>H]arachidonate levels in cells rendered permeable over three distinct ranges of [Ca<sup>2+</sup>]<sub>i</sub> in the presence and absence of W-7 (50 μM), another calmodulin inhibitor (13). As demonstrated in Table II there is a reduction in free [<sup>3</sup>H]arachidonate levels in the W-7-treated cells. The difference between control and W-7-treated cells is greater as the [Ca<sup>2+</sup>]<sub>i</sub> increases, a pattern consistent with the decrease in PGE<sub>2</sub> synthesis observed with compound 48/80 (Fig. 5). These experiments thus demonstrate that arachidonic acid release and PGE<sub>2</sub> production are direct functions of changes in [Ca<sup>2+</sup>]<sub>i</sub> and are calmodulin-dependent.

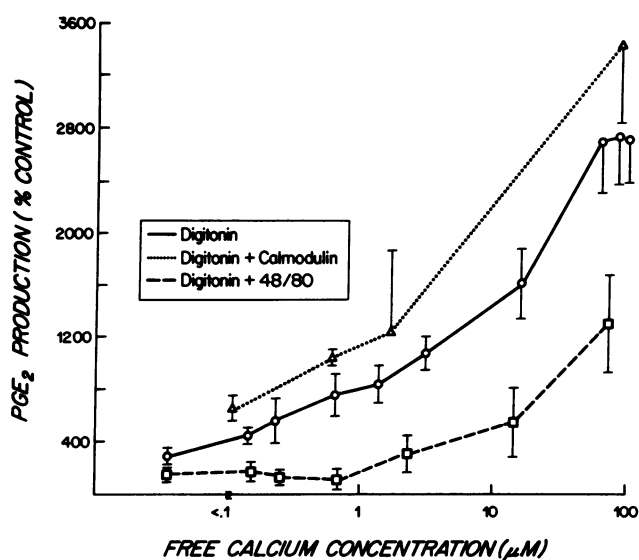


Figure 5. PGE<sub>2</sub> production as a function of free calcium concentration in cells made permeable with digitonin. Addition of calmodulin (5 μg/ml) had no significant effect on PGE<sub>2</sub> production. By contrast the calmodulin inhibitor, compound 48/80 (100 μg/ml), inhibited PGE<sub>2</sub> production over the entire range of Ca<sup>2+</sup> concentration tested. Each data point represents the mean±1 SE of 4–10 experiments. Control values represent the PGE<sub>2</sub> synthetic rates in the same plate of cells before addition of digitonin.

Table I. Free [<sup>3</sup>H]Arachidonic Acid and [<sup>3</sup>H]Diglyceride in Digitonin-treated Cells as a Function of [Ca<sup>2+</sup>]<sub>i</sub>

| Ca <sup>2+</sup> concentration | [% total radioactivity]           |                              |
|--------------------------------|-----------------------------------|------------------------------|
|                                | [ <sup>3</sup> H]Arachidonic acid | [ <sup>3</sup> H]Diglyceride |
| <100 nM                        | 1.7±0.3                           | 0.7±0.1                      |
| 1–10 μM                        | 5.4±1.9                           | 1.6±0.4                      |
| 500 μM                         | 14.9±7.0                          | 2.5±0.5                      |

Cells previously labeled with [<sup>3</sup>H]arachidonic acid were permeabilized in the presence of three different ranges of calcium concentration. The incubation was stopped after 10 min. Cells and supernatant were combined and assayed for free [<sup>3</sup>H]arachidonate and [<sup>3</sup>H]diglyceride by TLC after chloroform-methanol extraction (see Methods). Results represent the mean±SEM of four experiments. Each value of [<sup>3</sup>H]arachidonic acid and [<sup>3</sup>H]diglyceride was statistically different from values obtained at different [Ca<sup>2+</sup>]<sub>i</sub> ranges (*P* < 0.05).

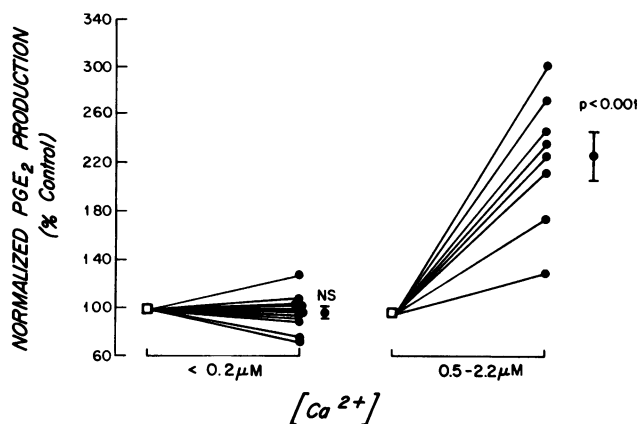
**Protein kinase C enhancement of PGE<sub>2</sub> synthesis and phospholipase A<sub>2</sub> activity.** In comparing Figs. 1, 2, and 5 it is clear that stimulated PGE<sub>2</sub> production in the permeabilized cell was lower than that found in the intact cell in the range of [Ca<sup>2+</sup>]<sub>i</sub> that is reached intracellularly after vasopressin stimulation. One explanation for this difference might be that other factors besides calcium itself may be operating in the vasopressin-stimulated intact cell. Since vasopressin, via its action to stimulate phospholipase C, also results in protein kinase C activation, permeabilized mesangial cells were studied in the presence and absence of a phorbol ester, PMA, a known stimulator of protein kinase C. The effect of 300 nM PMA on PGE<sub>2</sub> production was compared at two different ranges of [Ca<sup>2+</sup>]<sub>i</sub> (Fig. 6): resting cell levels, < 0.2 μM, and in the approximate range achieved in vasopressin-treated cells, between 0.5 and 2.2 μM (5). At basal [Ca<sup>2+</sup>]<sub>i</sub> there was no effect of PMA on PGE<sub>2</sub> synthesis. However, at Ca<sup>2+</sup> concentrations in the range achieved intracellularly with vasopressin-induced stimulation, PMA significantly enhanced PGE<sub>2</sub> production. PGE<sub>2</sub> production in PMA-stimulated permeabilized cells in the presence of 0.3–2.2 μM Ca<sup>2+</sup> concentration was equivalent to that in vasopressin-stimulated intact cells.

Table II. Effect of W-7 on [<sup>3</sup>H]Arachidonic Acid Release from Digitonin-treated Cells as a Function of [Ca<sup>2+</sup>]<sub>i</sub>

| Ca <sup>2+</sup> concentration | Free [ <sup>3</sup> H]Arachidonic acid |          |
|--------------------------------|--|----------|
|                                | Control                                | W-7      |
|                                | [% total radioactivity]                |          |
| <100 nM                        | 4.5±0.5                                | 3.6±0.6  |
| 1–5 μM                         | 10.3±0.6                               | 7.9±0.8* |
| 500 μM                         | 14.0±1.3                               | 8.2±0.9† |

Cells previously labeled with [<sup>3</sup>H]arachidonic acid were permeabilized in the presence or absence of W-7 (50 μM), in the presence of three different levels of calcium concentration. The incubation was stopped after 10 min. Cells and supernatant were combined and assayed for free [<sup>3</sup>H]arachidonate by TLC after chloroform-methanol extraction. Results represent means±SEM of four to eight experiments.

\* *P* < 0.01, † *P* < 0.005 when compared with control.



**Figure 6.** Effect of PMA (●) on PGE<sub>2</sub> production in cells made permeable with digitonin. Values of PGE<sub>2</sub> production in the presence of PMA are presented paired with their non-PMA-treated controls (□). PMA had no effect upon PGE<sub>2</sub> production when Ca<sup>2+</sup> concentration was fixed at levels approximating basal levels of cytosolic free Ca<sup>2+</sup> concentration as measured with fura-2. PMA increased PGE<sub>2</sub> production when free Ca<sup>2+</sup> concentration to which the permeabilized cells were exposed was in the range 0.3–2.2 μM. The total PGE<sub>2</sub> production under conditions where both [Ca<sup>2+</sup>]<sub>i</sub> was elevated and PMA was present was equivalent to that measured in vasopressin-stimulated intact cells.

To further demonstrate that an increase in [Ca<sup>2+</sup>]<sub>i</sub> is necessary, albeit not sufficient, to explain the level of acylhydrolase activity observed with vasopressin, we added vasopressin (100 nM) to permeabilized cells in the presence of 100 μM GTPγS in buffer where [Ca<sup>2+</sup>]<sub>i</sub> was fixed at levels below 100 nM. This resulted in a significant increase in [<sup>3</sup>H]diglyceride from control values of 1.55±0.14% of total radioactivity to 2.37±0.29% (n = 8, P < 0.01) indicating phospholipase C activation and presumably protein kinase C stimulation. There was no associated increase in free [<sup>3</sup>H]arachidonate (vasopressin, GTPγS treated = 105±7% of controls). Thus, an increase in protein kinase C activity, under conditions where [Ca<sup>2+</sup>]<sub>i</sub> was held at low levels, was insufficient to increase acylhydrolase activity. An increase in [Ca<sup>2+</sup>]<sub>i</sub> was necessary.

To provide further evidence that the Ca<sup>2+</sup> dependent acylhydrolase activity in permeabilized cells was due to activation of phospholipase A<sub>2</sub>, the effects of two phospholipase A<sub>2</sub> inhibitors on arachidonic acid release and diglyceride levels were determined. As demonstrated in Table III both dibucaine and mepacrine significantly reduced levels of free [<sup>3</sup>H]arachidonic acid at all levels of Ca<sup>2+</sup> concentrations. By contrast neither agent had any effect on [<sup>3</sup>H]diglyceride levels indicating no effect on phospholipase C.

To validate that this apparent synergy of action between Ca<sup>2+</sup> and protein kinase C activation on phospholipase A<sub>2</sub> activation also occurs in the nonpermeabilized cell we measured arachidonic acid release in response to A23187 (0.1 μM), PMA (300 nM), A23187 (0.1 μM) together with PMA (300 nM) and vasopressin (100 nM) alone. As summarized in Fig. 7, A23187 at this low dose resulted in a slight increase in arachidonic acid release as would be expected from an increase in [Ca<sup>2+</sup>]<sub>i</sub> due to the ionophore (Δ[Ca<sup>2+</sup>]<sub>i</sub> = 200–300 nM as determined with 4-Br A23187, a nonfluorescent analogue of A23187). PMA had no effect on arachidonic acid release, and

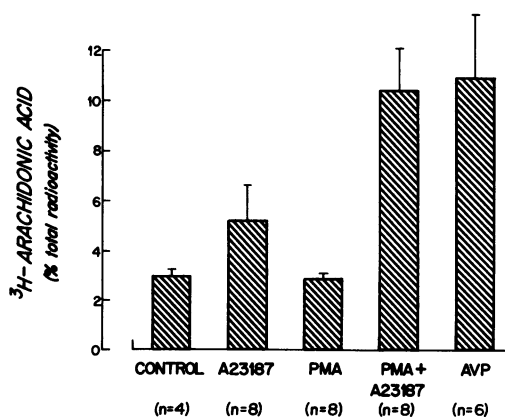
**Table III.** Effects of Dibucaine and Mepacrine on Free [<sup>3</sup>H]Arachidonic Acid and [<sup>3</sup>H]Diglyceride in Digitonin-treated Cells as a Function of [Ca<sup>2+</sup>]<sub>i</sub>

| Ca <sup>2+</sup> concentration | Condition | [ <sup>3</sup> H]Arachidonic acid | [ <sup>3</sup> H]Diglyceride |
|--------------------------------|-----------|-----------------------------------|------------------------------|
| % total radioactivity          |           |                                   |                              |
| <100 nM                        | Control   | 1.44±0.10                         | 0.45±0.02                    |
|                                | Dibucaine | 0.77±0.01*                        | 0.48±0.04                    |
|                                | Control   | 1.58±0.06                         | 0.38±0.02                    |
|                                | Mepacrine | 0.91±0.07*                        | 0.41±0.02                    |
| 1 μM                           | Control   | 2.04±0.17                         | 0.72±0.04                    |
|                                | Dibucaine | 1.21±0.09‡                        | 0.95±0.07                    |
|                                | Control   | 2.10±0.11                         | 0.67±0.12                    |
|                                | Mepacrine | 1.29±0.06*                        | 0.65±0.05                    |
| 500 μM                         | Control   | 12.72±1.20                        | 2.28±0.09                    |
|                                | Dibucaine | 2.98±0.52*                        | 2.92±0.13                    |
|                                | Control   | 15.05±1.18                        | 2.18±0.15                    |
|                                | Mepacrine | 8.20±0.63*                        | 2.42±0.10                    |

Cells previously labeled with [<sup>3</sup>H]arachidonic acid were permeabilized in the presence or absence of a phospholipase A<sub>2</sub> inhibitor in the presence of three different levels of calcium concentration. The concentration of dibucaine and mepacrine were 250 and 100 μM, respectively. Results represent mean±SEM of seven to eight experiments.

\* P < 0.001, ‡ P < 0.005 when compared with respective controls. Dibucaine and mepacrine had no significant effect on cellular [<sup>3</sup>H]diglyceride levels at any of the three levels of [Ca<sup>2+</sup>]<sub>i</sub> tested.

no effect on [Ca<sup>2+</sup>]<sub>i</sub> under these conditions (Fig. 8). Although the increase in [Ca<sup>2+</sup>]<sub>i</sub> observed with ionophore was unaffected by simultaneous addition of PMA (300 μM) PMA markedly enhanced arachidonic acid release. The levels of free arachidonic acid observed with A23187 and PMA together were



**Figure 7.** Effect of 10 min of stimulation of nonpermeabilized cells with the Ca<sup>2+</sup> ionophore, A23187 (0.1 μM) and PMA (300 nM) on [<sup>3</sup>H]arachidonic acid release from prelabeled cells. At this low concentration of A23187 there was little stimulation of arachidonic acid release. PMA alone had no effect. However, PMA together with A23187 stimulated the release of arachidonic acid to levels observed with vasopressin. Each data bar represents the mean±1 SEM of six to eight experiments.

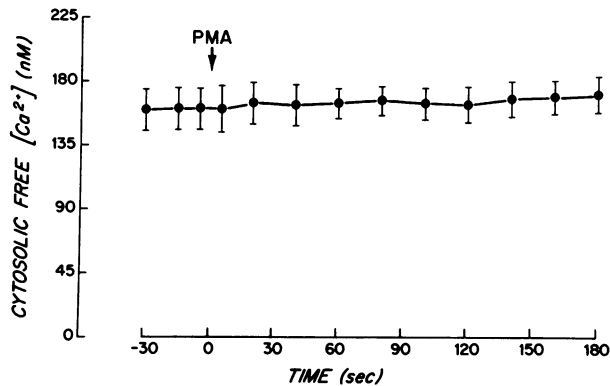


Figure 8. Lack of an effect of PMA (300 nM) on  $[Ca^{2+}]_i$ .  $[Ca^{2+}]_i$  was measured using fura-2 as described in Methods. Results represent data derived from six experiments.

equivalent to the levels observed with vasopressin alone. Experiments were also performed with higher doses of A23187 (1  $\mu$ M) over longer incubation periods (30 min) to determine whether higher levels of  $[Ca^{2+}]_i$  would result in levels of acyl-hydrolase activation observed with vasopressin. At this dose of A23187,  $[Ca^{2+}]_i$  was increased to > 1  $\mu$ M. As indicated in Fig. 9, even with the increased dose and time of incubation, A23187 increased free  $[^3H]$ arachidonate levels to a much less extent than AVP. In the presence of the higher dose of A23187, however, low doses of PMA (10 nM) resulted in  $[^3H]$ arachidonate release that was considerably greater than that observed with vasopressin. As demonstrated in Table IV, 1-oleoyl 2-acetyl-glycerol, a diacylglycerol analogue, also acted synergistically with A23187 to increase free arachidonate levels in intact mesangial cells.

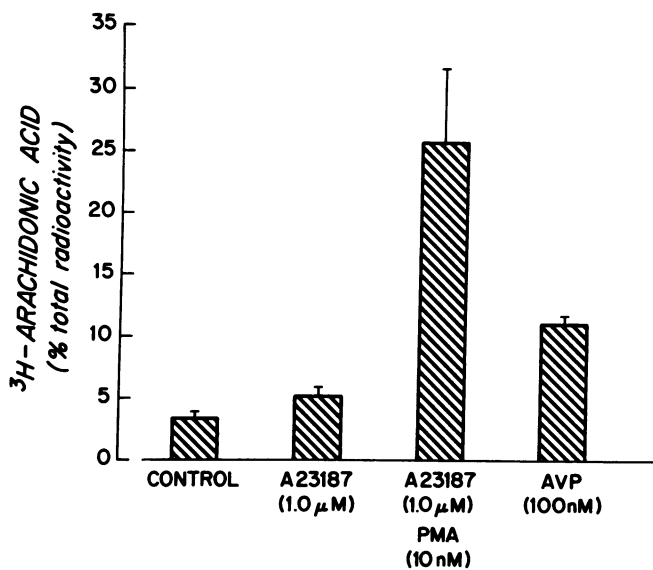


Figure 9. Effect of 30 min of stimulation of nonpermeabilized cells with the  $Ca^{2+}$  ionophore, A23187 (1.0  $\mu$ M) and PMA (10 nM) on  $[^3H]$ arachidonic acid release from prelabeled cells. The dose of A23187 was higher and that of PMA lower than the doses used in experiments depicted in Fig. 7. Even at this low dose of PMA there was marked enhancement of the A23187-induced increase in arachidonic acid release to levels exceeding that of vasopressin alone. Each data bar represents the mean  $\pm$  1 SEM of three to six experiments.

Table IV. Effect of 1-Oleoyl 2-Acetyl-glycerol on  $[^3H]$ Arachidonic Acid Release from Intact Mesangial Cells

| Treatment                           | Free $[^3H]$ Arachidonic acid<br>% control |
|-------------------------------------|--|
| A23187                              | 106 $\pm$ 14                               |
| 1-Oleoyl 2-acetyl-glycerol          | 117 $\pm$ 15                               |
| A23187 + 1-oleoyl 2-acetyl-glycerol | 148 $\pm$ 14 <sup>A</sup>                  |

Cells previously labeled with  $[^3H]$ arachidonic acid were treated with A23187, 1-oleoyl 2-acetyl-glycerol or the combination of both agents. After 10 min free  $[^3H]$ arachidonic acid was determined in cell and supernatant. The concentrations of A23187 and 1-oleoyl 2-acetyl-glycerol were 0.1  $\mu$ M and 100  $\mu$ g/ml, respectively. Results represent the means  $\pm$  SEM of 10 experiments.

<sup>A</sup>  $P < 0.01$  compared to control.

## Discussion

We have previously demonstrated that vasopressin increases  $[Ca^{2+}]_i$  in glomerular mesangial cells (5).  $[Ca^{2+}]_i$  is increased primarily due to release from intracellular storage sites rather than entry of  $Ca^{2+}$  from the extracellular environment. This is consistent with our present observations that PGE<sub>2</sub> production was not dependent upon extracellular  $[Ca^{2+}]_i$ . Our results are at variance with those of Scharschmidt and Dunn (14) who found that removal of extracellular  $Ca^{2+}$  completely inhibited AVP-induced PGE<sub>2</sub> synthesis. The difference in results is likely explained by the fact that Scharschmidt and Dunn equilibrated the mesangial cells for 1 h in a "no-added  $Ca^{2+}$ " solution before the addition of AVP, whereas we exposed cells to low  $[Ca^{2+}]_i$  for 10 min or less, prior to addition of AVP. This likely resulted in depletion of intracellular  $Ca^{2+}$  stores with resultant loss of the ability of vasopressin to increase  $[Ca^{2+}]_i$ .

While several laboratories have found that phospholipase A<sub>2</sub> activity in isolated cellular extracts is  $Ca^{2+}$  dependent, the concentrations of  $Ca^{2+}$  used to stimulate enzymatic activity are well above those measured in intact cells (1, 2). In sheep erythrocyte membranes no phospholipase A<sub>2</sub> activity was observed when  $Ca^{2+}$  concentration was less than 10  $\mu$ M and one-half maximal activity was found at  $Ca^{2+}$  concentrations of 100–500  $\mu$ M (1). When cells are disrupted and membrane fractions or isolated enzymes are assayed phospholipase A<sub>2</sub> activity may be altered for many reasons. Important cofactors or modulating factors may be lost or altered. Endogenous inhibitors may be activated (3). By using a permeable cell less perturbation of the microenvironment of the phospholipase enzyme might occur. In addition, other cellular factors potentially critical to phospholipase A<sub>2</sub> activity, such as calmodulin, would be present in the permeabilized cell. Our experiments demonstrate that arachidonic acid release and PGE<sub>2</sub> synthesis are stimulated when  $[Ca^{2+}]_i$  is increased over the range of  $[Ca^{2+}]_i$  observed in cells stimulated with physiological agonists that lead to prostaglandin production; however, the increase observed with  $[Ca^{2+}]_i$  is insufficient to explain the activation seen with vasopressin in intact cells. The inhibition of  $Ca^{2+}$ -stimulated PGE<sub>2</sub> production with compound 48/80 and acyl-hydrolase activity with W-7 is consistent with a calmodulin dependency of phospholipase A<sub>2</sub> activation and PGE<sub>2</sub> production (15).

The experiments performed with TMB-8 demonstrate both an inhibition of the vasopressin-induced increase in



$[Ca^{2+}]_f$  and an associated decreased PGE<sub>2</sub> production. While TMB-8 has been proposed to block Ca<sup>2+</sup> release from intracellular stores, in only a few cases (e.g., 16) has it been shown to actually modify the pattern of agonist-induced Ca<sup>2+</sup> transients and its effects on Ca<sup>2+</sup> metabolism have not previously been examined in the mesangial cell. Our data demonstrate, however, that the inhibition of vasopressin-induced PGE<sub>2</sub> synthesis induced by TMB-8 in the intact cell was likely due, not only to a decrease in the vasopressin-induced  $[Ca^{2+}]_f$  response, but also a  $[Ca^{2+}]_f$ -independent effect of the TMB-8. This later effect was established by the action of TMB-8 to inhibit PGE<sub>2</sub> synthesis in permeabilized preparations where the  $[Ca^{2+}]_f$  was clamped and may be due to inhibition of phospholipase A<sub>2</sub>.

The stimulation of PGE<sub>2</sub> by PMA in the presence of increased  $[Ca^{2+}]_f$  suggests that protein kinase C activation can modulate mesangial cell acylhydrolase activity since protein kinase C is the cellular receptor for this phorbol ester (17, 18). The pattern of stimulation was of particular interest since the PMA only stimulated PGE<sub>2</sub> production if the cytosolic Ca<sup>2+</sup> concentration was raised above baseline values. In pituitary cells a phorbol ester-induced stimulation of PGE<sub>2</sub> release was reported to be "Ca<sup>2+</sup> dependent" (19); however, this conclusion was derived from experiments performed in the presence or absence of added extracellular Ca<sup>2+</sup> for periods of 5 h. No attempt was made to correlate extracellular Ca<sup>2+</sup> with intracellular Ca<sup>2+</sup> concentration. It has also been shown, for a variety of other cellular responses, that protein kinase C activation and  $[Ca^{2+}]_f$  act synergistically (20, 21). Depending upon the system studied protein kinase C stimulation alone, in the absence of an increase in  $[Ca^{2+}]_f$ , may be sufficient to increase phospholipase A<sub>2</sub> activity but requires much longer periods of stimulation (22, 23).

The synergistic interaction between increases in  $[Ca^{2+}]_f$  and protein kinase C activation was also manifest in our experiments in which arachidonic acid release was measured after A23187 and PMA were added to nonpermeabilized cells. PMA alone had no effect. When  $[Ca^{2+}]_f$  was increased with A23187, however, the addition of PMA markedly enhanced arachidonate release as compared with release measured with A23187 alone. Likewise 1-oleoyl 2-acetyl-glycerol, a cell permeant diacylglycerol analogue, acts synergistically with A23187 to enhance acylhydrolase activity, albeit with lower potency than PMA. These data suggest that a protein kinase C dependent phosphorylation process enhances acylhydrolase activity only if there is a simultaneous increase in  $[Ca^{2+}]_f$ .

Our data indicate that the Ca<sup>2+</sup> and protein kinase C stimulated acylhydrolase activity in mesangial cells is primarily due to phospholipase A<sub>2</sub>. Two different phospholipase A<sub>2</sub> inhibitors, dibucaine and mepacrine, inhibit the Ca<sup>2+</sup>-dependent acylhydrolase activity without altering phospholipase C activity. Furthermore, stimulation of phospholipase C with GTP $\gamma$ S and vasopressin in permeabilized preparations under conditions where  $[Ca^{2+}]_f$  is maintained at low levels (< 100 nM), results in no stimulation of arachidonic acid release. Under these conditions diacylglycerol and presumably phosphatidic acid are increased providing enhanced substrate for diacylglycerol lipase or phosphatidic acid specific acylhydrolase enzymes. The associated absence of enhanced acylhydrolase activity indicates that phospholipase A<sub>2</sub> is likely the primary enzymatic source of acylhydrolase activity in the mesangial cell under our experimental conditions. This is consistent with the findings of Schlondorff et al. (24) who also find that diacyl-

glycerol lipase plays a minimal role in arachidonic acid release in the mesangial cell. Ho and Klein (25) recently suggested that protein kinase C may play an important role in the Ca<sup>2+</sup>-induced stimulation of phospholipase A<sub>2</sub> in the pineal gland.

One explanation for the potentiation of Ca<sup>2+</sup>-dependent phospholipase A<sub>2</sub> activity by protein kinase C may be that protein kinase C phosphorylates phospholipase A<sub>2</sub> itself or protein(s) with phospholipase A<sub>2</sub> modulatory capability such as lipocortin (26). It has been proposed that lipocortin loses phospholipase A<sub>2</sub> inhibitory activity when phosphorylated, resulting in increased phospholipase A<sub>2</sub> activity and enhanced PGE<sub>2</sub> production (27). In collaboration with Dr. B. Pepinsky we have documented the presence of two proteins that have been cloned, sequenced and named "lipocortin I" and "lipocortin II" by Pepinsky and colleagues (28), as well as the respective mRNAs, in mesangial cells. However, there remains considerable controversy as to whether these proteins represent endogenous functionally regulated inhibitors of phospholipase A<sub>2</sub> (29). There is evidence for modulation of phospholipase A<sub>2</sub> by phosphorylation in other systems. Moskowitz et al. (30) demonstrated enhanced phospholipase A<sub>2</sub> activity by addition of Mg<sup>2+</sup>, ATP, and cAMP to brain synaptosomal vesicles. Wightman et al. (31) showed a similar activation by ATP in mouse peritoneal macrophage sonicates and further demonstrated that cAMP-dependent protein kinase augmented phospholipase A<sub>2</sub> activation. Another possible explanation for the enhanced phospholipase A<sub>2</sub> activity with both an increase in  $[Ca^{2+}]_f$  and protein kinase C activity is that the subtype of protein kinase C stimulated by PMA and 1-oleoyl 2-diacylglycerol, and hence the substrate specificity, may be altered by the simultaneous increase in cytosolic  $[Ca^{2+}]_f$  (32).

Alternatively, protein kinase C may phosphorylate a GTP binding protein which enhances acylhydrolase activity when the latter is stimulated by Ca<sup>2+</sup>. In FRTL-5 rat thyroid cells, phospholipase A<sub>2</sub> is modulated by a pertussis-toxin-sensitive G-protein, whereas phospholipase C is regulated by a different G-protein (33). Light-stimulated phospholipase A<sub>2</sub> activity in rod outer segments of bovine retina is modulated by a transducin-like G-protein (34). Phospholipase A<sub>2</sub> activity in RAW 264.7 macrophages is enhanced by both cholera toxin and pertussis toxin, suggesting G<sub>s</sub> and G<sub>i</sub> involvement in the process (35). Finally, in the mesangial cell, Schlondorff et al. (36) and Pfeilschifter and Bauer (37) have found pertussis toxin modulation of agonist-induced PGE<sub>2</sub> production. Our studies, which show no effect of GTP  $\gamma$ S on acylhydrolase activity when  $[Ca^{2+}]_f$  is clamped < 100 nM, do not rule out a G-protein mediated potentiation of Ca<sup>2+</sup> dependent phospholipase A<sub>2</sub> activation which can be observed only at higher levels of  $[Ca^{2+}]_f$ .

Protein kinase C activation could potentially activate a Na<sup>+</sup>/H<sup>+</sup> exchange process (38) that we have identified in the mesangial cell (39). This in turn may activate phospholipase A<sub>2</sub> by a phospholipase C independent mechanism as has been reported in the platelet (40). This could not, however, explain the PMA induced increase in PGE<sub>2</sub> synthesis in permeabilized cells since pH as well as Ca<sup>2+</sup> is fixed under these conditions.

In summary, our studies demonstrate the role played by Ca<sup>2+</sup> in prostaglandin synthesis in the mesangial cell. There are at least two interrelated mechanisms for activation of PGE<sub>2</sub> production in response to hormonal agonists such as vasopressin. The elevation in cytosolic free  $[Ca^{2+}]_f$  stimulates phospholipase A<sub>2</sub> activity but is not sufficient to explain the mea-



sured PGE<sub>2</sub> production. The activation of protein kinase C, which occurs secondary to agonist-mediated phospholipase C activation and diacylglycerol production, further enhances the production of PGE<sub>2</sub> by further increasing Ca<sup>2+</sup>-dependent phospholipase A<sub>2</sub> activity. The mechanisms accounting for these synergistic interactions between Ca<sup>2+</sup> and protein kinase C activation are incompletely understood at present but may involve the phosphorylation of phospholipase A<sub>2</sub> or endogenous phospholipase A<sub>2</sub> modulatory proteins.

## Acknowledgments

The excellent technical help of Karen Dellovo and secretarial help of Lisa Firicano are gratefully acknowledged.

These studies were supported by National Institutes of Health grants HL-31513, DK-39773, and DK-38452, and an individual National Research Service Award HL-07015 to Mark Swidler. J. V. Bonventre is an Established Investigator of the American Heart Association.

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